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(54) Title: METHOD OF TREATING TUMOR CELLS BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

(57) Abstract

A method of inhibiting growth of tumor cells which overexpress a growth factor receptor or growth factor by treatment of the cells with antibodies which inhibit the growth factor receptor function is disclosed. A method of treating tumor cells with antibodies which inhibit growth factor receptor function, and with cytotoxic factor(s) such as tumor necrosis factor, is also disclosed. By inhibiting growth factor receptor functions tumor cells are rendered more susceptible to cytotoxic factors.

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METHOD OF TREATING TUMOR CELLS BY INHIBITING GROWTH FACTOR RECEPTOR FUNCTION

Field of the Invention

This invention is in the fields of immunology and cancer diagnosis and therapy. More particularly it concerns antibodies specifically binding growth factor receptors, hybridomas that produce these antibodies, immunochemicals made from the antibodies, and diagnostic methods that use the antibodies. The invention also relates to the use of the antibodies alone or in combination with cytotoxic factor(s) in therapeutic methods. Also encompassed by the invention is an assay for tyrosine kinases that are involved in tumorigenesis.

Background of the Invention

Macrophages are one of the effector cell types that play an important role in immunosurveillance against neoplastic growth *in vivo*. *In vitro*, cell-mediated cytotoxicity requires selective binding between activated macrophages and target cells as well as the concomitant release of cytotoxic factors. Some of the cytotoxic factors secreted by activated macrophages include reactive oxygen species such as the superoxide anion and hydrogen peroxide, arginase, interleukin 1, and tumor necrosis factor-α (TNF-α). Acquired resistance to the toxic effects of these factors by tumor cells could be one mechanism which leads to the onset and spread of tumor formation *in vivo*.

The observation that TNF-α can act as a potent effector of the macrophage-mediated antitumor response provides a rationale for its use in further studies on the regulation of tumorigenesis *in vivo* and tumor cell growth *in vitro*. The genes encoding TNF-α and TNF-β, a structurally related cytotoxic protein formerly known as lymphotoxin, have been cloned and the corresponding proteins expressed in *Escherichia coli*. These proteins display an array of biological activities, including induction of hemorrhagic necrosis

of Meth A sarcomas in vivo, inhibition of the growth of certain tumor cells in vitro, synergistic enhancement of the in vitro anticellular effects of IFN-γ, activation of human polymorphonuclear neutrophil functions, and inhibition of lipid biosynthesis. Recently, rHuTNF-α was shown to augment the growth of normal diploid fibroblasts in vitro. The divergent proliferative responses in the presence of rHuTNF-α are sometimes related to variations in TNF binding.

Growth factors and their receptors are involved in the regulation of cell proliferation and they also appear to play a key role in oncogenesis. Of the known proto-oncogenes, three are related to a growth factor or a growth factor receptor. These genes include *c-sis*, which is homologous to the transforming gene of the simian sarcoma virus and is the B chain of platelet-derived growth factor (PDGF); *c-fms*, which is homologous to the transforming gene of the feline sarcoma virus and is closely related to the macrophage colony-stimulating factor receptor (CSF-1R); an *c-erb*B, which encodes the EGF receptor (EGFR) and is homologous to the transforming gene of the avian erythroblastosis virus (*v-erb*B). The two receptor-related proto-oncogenes, *c-fms* and *c-erb*B, are members of the tyrosine-specific protein kinase family to which many proto-oncogenes belong.

Recently, a novel transforming gene was identified as a result of transfection studies with DNA from chemically induced rat neuroblastomas. This gene, called *neu*, was shown to be related to, but distinct from, the *c-erb*B proto-oncogene. By means of *v-erb*B and human EGFR as probes to screen human genomic and complementary DNA (cDNA) libraries, two other groups independently isolated human *erb*B-related genes that they called HER2 and *c-erb*B-2 respectively. Subsequent sequence analysis and chromosomal mapping studies revealed that *c-erb*B-2, and HER2 are species variants of *neu*. A fourth group, also using *v-erb*B as a probe, identified the same

gene in a mammary carcinoma cell line, MAC 117, where it was found to be amplified five- to ten-fold.

This gene, which will be referred to herein as HER2, encodes a new member of the tyrosine kinase family; and is closely related to, but distinct from, the EGFR gene as reported by Coussens et al., Science 230, 1132 (1985). HER2 differs from EGFR in that it is found on band q21 of chromosome 17, as compared to band p11-p13 of chromosome 7, where the EGFR gene is located. Also, the HER2 gene generates a messenger RNA (mRNA) of 4.8 kb, which differs from the 5.8- and 10-kb transcripts for the EGFR gene. Finally, the protein encoded by the HER2 gene is 185,000 daltons, as compared to the 170,000-dalton protein encoded by the EGFR gene. Conversely, on the basis of sequence data, HER2 is more closely related to the EGFR gene than to other members of the tyrosine kinase family. Like the EGFR protein, the HER2 protein (p185) has an extracellular domain, a transmembrane domain that includes two cysteine-rich repeat clusters, and an intracellular kinase domain, indicating that it is likely to be a cellular receptor for an as yet unidentified ligand. HER2 p185 is referred to as p185 or the HER2 receptor herein.

Southern analysis of primary human tumors and established tumor-derived cell lines revealed amplification and in some cases rearrangement of the EGF receptor gene. Amplification was particularly apparent in squamous carcinomas and glioblastomas. The HER2 gene was also found to be amplified in a human salivary gland adenocarcinoma, a renal adenocarcinoma, a mammary gland carcinoma, and a gastric cancer cell line. Recently, Slamon et al., Science 235, 177 (1987) demonstrated that about 30% of primary human breast carcinoma tumors contained an amplified HER2 gene. Although a few sequence rearrangements were detected, in most tumors there were no obvious differences between amplified and normal HER2 genes. Furthermore, amplification of the HER2 gene

correlated significantly with the negative prognosis of the disease and the probability of relapse.

To investigate the significance of the correlation between over-expression and cellular transformation as it has been observed for proto-oncogenes *c-mos* and N-*myc*, a HER2 expression vector and a selection scheme that permitted sequence amplification after transfection of mouse NIH 3T3 cells was employed by Hudziak et al., Proc. Natl. Acad. Sci. (USA) 84, 7159 (1987). Amplification of the unaltered HER2 gene in NIH 3T3 cells lead to over-expression of p185 as well as cellular transformation and tumor formation in athymic mice.

The effects of antibodies specifically binding growth factors or growth factor receptors has been studied. Examples are discussed below.

Rosenthal et al., Cell 46, 301 (1986) introduced a human TGF-α cDNA expression vector into established non-transformed rat fibroblast cells. Synthesis and secretion of TGF-α by these cells resulted in loss of anchored-dependent growth and induced tumor formation in nude mice. Anti-human TGF-α monoclonal antibodies prevented the rat cells from forming colonies in soft agar, i.e. loss of anchorage dependence. Gill et al., in J. Biol. Chem. 259 7755 (1984) disclose monoclonal antibodies specific for EGF receptor which were inhibitors of EGF binding and antagonists of EGF-stimulated tyrosine protein kinase activity.

Drebin et al, in Cell 41 695 (1985) demonstrated that exposure of a *neu*-oncogene-transformed NIH 3T3 cell to monoclonal antibodies reactive with the *neu* gene product, cause the *neu*-transformed NIH 3T3 cell to revert to a non-transformed phenotype as determined by anchorage independent growth. Drebin et al., in Proc. Natl. Acad. Sci. 83, 9129 (1986) demonstrated that in vivo treatment with a monoclonal antibody (IgG2a isotype) specifically binding the

protein encoded by the *neu* oncogene significantly inhibited the tumorigenic growth of *neu*-transformed NIH 3T3 cells implanted into nude mice.

Akiyama et al. in Science 232, 1644 (1986) raised antibodies against a synthetic peptide corresponding to 14 amino acid residues at the carboxy-terminus of the protein deduced from the *c-erb*B-2 (HER2) nucleotide sequence.

Growth factors have been reported to interact in both a synergistic and an antagonistic manner. For example, TGF-α and TGF-β synergistically enhance the growth of NRK-49F fibroblasts, whereas PDGF down regulates EGF receptor function on 3T3 cells. A variety of transformed cells secrete factors which are believed to stimulate growth by an autocrine mechanism. Sugarman et al., Cancer Res. 47, 780 (1987) demonstrated that under certain conditions, growth factors can block the antiproliferative effects of TNF-α on sensitive tumor cells. Specifically, epidermal growth factor (EGF) and recombinant human transforming growth factor-α (rHuTGF-α) were shown to interfere with the in vitro antiproliferative effects of recombinant human tumor necrosis factor-α (rHuTNF-α) and -β on a human cervical carcinoma cell line, ME-180. The inhibitory effect could be observed at EGF or rHuTGF-α concentrations of 0.1 to 100 ng/ml, and was maximal between 1 and 10 ng/ml. This response was apparently not due to down regulation of the TNF receptor or to alteration of the affinity of TNF-α for its receptor. Since the antiproliferative effect of recombinant human interferon-γ was not significantly affected by the presence of EGF or rHuTGF-α, the inhibition was specific for recombinant TNFs and was not due solely to enhanced proliferation induced by the growth factors. Neither growth factor had a substantial protective effect on the synergistic cytotoxicity observed when tumor cells were exposed simultaneously to rHuTNF-α and recombinant human interferon-γ. TGF-β can also interfere with the antiproliferative effects of rHuTNF-α in vitro. At concentrations

of less than 1 ng/ml, TGF-β significantly antagonized the cytotoxic effects of rHuTNF-α on NIH 3T3 fibroblasts. Since EGF, platelet-derived growth factor, and TGF-β all enhanced NIH 3T3 cell proliferation, but only TGF-β interfered with rHuTNF-α cytotoxicity, the protective effects of TGF-β were not related in a simple manner to enhanced cell proliferation. rHuTGF-α and TGF-β did not have a significant protective effect against rHuTNF-α-mediated cytotoxicity on two other tumor cell lines, BT-20 and L-929 cells.

It is an object of the subject invention to provide antibodies capable of inhibiting growth factor receptor function.

It is a further object of the invention to provide an improved assay for the HER2 receptor.

It is a further object of the invention to provide improved methods of tumor therapy.

It is a further object of the invention to provide a method of inhibiting the growth of tumor cells which overexpress a growth factor receptor and/or growth factor.

It is a further object of the invention to provide a method for treating a tumor by treatment of the tumor cells with antibodies capable of inhibiting growth factor receptor function, and with cytotoxic factors such as tumor necrosis factor.

A still further object of the invention is to provide an assay for tyrosine kinases that may have a role in tumorigenesis.

Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

Summary of the Invention

The subject invention relates to monoclonal antibodies specifically binding the external domain of the HER2 receptor. The invention also relates to an assay for the HER2 receptor comprising exposing cells to antibodies specifically binding the extracellular domain of the HER2 receptor, and determining the extent of binding of said antibodies to said cells. Another embodiment of the invention relates to a method of inhibiting growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies capable of inhibiting the HER2 receptor function. A further embodiment of the invention relates to administering a therapeutically effective amount of antibodies capable of inhibiting growth factor receptor function, and a therapeutically effective amount of a cytotoxic factor. A still further embodiment of the invention is an assay for tyrosine kinases that may have a role in tumorigenesis comprising exposing cells suspected to be TNF-α resistant to TNF-α, isolating those cell which are TNF-α resistant, screening the isolated cells for increased tyrosine kinase activity, and isolating receptors and other proteins having increased tyrosine kinase activity.

Brief Description of the Drawings

Figure 1a shows TNF-α resistance of NIH 3T3 cells expressing various levels of HER2 p185. Figure 1b shows macrophage cytotoxicity assays for NIH 3T3 cells expressing various levels of HER2 p185.

Figure 2 demonstrates the level of TNF-α binding for a control cell line (NIH 3T3 neo/dhfr) and for a cell line overexpressing HER2 p185 (HER2-3_{800}).

Figure 3 shows inhibition of SK BR3 cell growth by anti-HER2 monoclonal antibodies.

Figure 4 is a dose response curve comparing the effect of an irrelevant monoclonal antibody (anti-HBV) and the effect of monoclonal antibody 4D5 (anti-HER2) on the growth of SK BR3 cells in serum.

Figures 5a, 5b and 6a show percent viability of SK BR3 cells as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration. Each Figure shows the results for a different anti-HER2 p185 monoclonal antibody. Figure 6b is a control using an irrelevant monoclonal antibody.

Figure 7 shows percent viability of MDA-MB-175-VII cells as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration.

Figure 8 shows percent viability of NIH 3T3 cells overexpressing HER2 p185 as a function of increasing TNF-α concentration and anti-HER2 p185 monoclonal antibody concentration.

<u>Detailed Description of the Invention</u>

A new application of antibodies to inhibit the growth of tumor cells has been discovered. Surprisingly, it has been found that by inhibiting growth factor receptor function, e.g. the HER2 receptor function, cell growth is inhibited, and the cells are rendered more susceptible to cytotoxic factors. Thus, for example, breast cancer cells which are refractory to TNF-α alone can be made susceptible to TNF-α if the cells are first treated with antibodies which inhibit growth factor receptor function. The increase of susceptibility has been demonstrated using the HER2 receptor and monoclonal antibodies directed against the HER2 receptor, and tumor necrosis factor-α.

The method of this invention is useful in the therapy of malignant or benign tumors of mammals where the abnormal growth rate of the tumor is dependent upon growth factor receptors.

Abnormal growth rate is a rate of growth which is in excess of that required for normal homeostasis and is in excess of that for normal tissues of the same origin. Many of these tumors are dependent upon extracellular sources of the growth factor recognized by the receptor, or upon synthesis of the growth factor by the tumor cell itself. This latter phenomenon is termed "autocrine" growth.

The methods of the subject invention is applicable where the following conditions are met:

(1) the growth factor receptor and/or ligand (growth factor) is expressed, and tumor cell growth depends upon the growth factor receptor biological function;

(2) antibodies specifically binding the growth factor receptor and/or ligand inhibit the growth factor receptor biological function.

While not wishing to be constrained to any particular theory of operation of the invention, it is believed that the antibodies inhibit growth factor receptor biological function in one or more of the following ways:

(a) The antibodies bind to the extracellular domain of the receptor and inhibit the ligand from binding the receptor;

(b) The antibodies bind the ligand (the growth factor) itself and inhibit the ligand from binding the receptor;

(c) The antibodies down regulate the growth factor receptor;

(d) The antibodies sensitize tumor cells to the cytotoxic effects of a cytotoxic factor such as TNF-α;

(e) The antibodies inhibit the tyrosine kinase activity of the receptor.

In cases (f) and (g), the antibodies inhibit growth factor receptor biological function indirectly by mediating cytotoxicity via a targeting function:

(f) The antibodies belong to a sub-class or isotype that upon complexing with the receptor activates serum complement and/or

mediate antibody-dependent cellular cytotoxicity (ADCC), e.g. IgG2a antibodies;

(g) The antibodies which bind the receptor or growth factor are conjugated to a toxin (immunotoxins);

Advantageously antibodies are selected which greatly inhibit the receptor function by binding the steric vicinity of the ligand binding site of the receptor (blocking the receptor), and/or which bind the growth factor in such a way as to prevent (block) the ligand from binding to the receptor. These antibodies are selected using conventional in vitro assays for selecting antibodies which neutralize receptor function. Antibodies that act as ligand agonists by mimicking the ligand are discarded by conducting suitable assays as will be apparent to those skilled in the art. For certain tumor cells, the antibodies inhibit an autocrine growth cycle (i.e. where a cell secretes a growth factor which then binds to a receptor of the same cell). Since some ligands, e.g. TGF-α, are found lodged in cell membranes, the antibodies serving a targeting function are directed against the ligand and/or the receptor.

Certain tumor cells secrete growth factors that are required for normal cellular growth and division. These growth factors, however, can under some conditions stimulate unregulated growth of the tumor cell itself, as well as adjacent non-tumor cells, and can cause a tumor to form.

Epidermal Growth Factor (EGF) has dramatic stimulatory effects on cell growth. In purified receptor preparations, the EGF receptor is a protein kinase that is activated by the binding of EGF. Substrate proteins for this kinase are phosphorylated on tyrosine residues. The receptors for insulin, platelet-derived growth factor (PDGF) and other growth hormones also are tyrosine-specific kinases. It is believed that ligand binding to the receptor triggers phosphorylation of certain proteins by the receptor and in this way stimulates cell growth. About one-third

of the known oncogenes encode proteins that phosphorylate tyrosine residues on other proteins. It is believed that these oncogene products trigger responses analogous to the responses of cells to growth factors and hormones. The *erb*B oncogene product is a portion of the EGF receptor that lacks the hormone-binding domain and may give rise to a constitutive growth-stimulating signal.

One embodiment of this invention is a method of inhibiting the growth of tumor cells by administering to a patient a therapeutically effective amount of antibodies that inhibit the HER2 receptor biological function of tumor cells.

Overexpression of growth factor receptors increases the resistance of cells to TNF as demonstrated below. Overexpression of the HER1 receptor (EGF receptor), met receptor-like protooncogene product, and HER2 receptor all show this increased resistance. It is shown in the Examples below that amplified expression of HER2, which encodes the HER2 receptor (p185), induces resistance of NIH 3T3 cells to the cytotoxic effects of macrophages or TNF-α. Induction of NIH 3T3 cell resistance to TNF-α by overexpression of p185 is accompanied by alterations in the binding of TNF-α to its receptor. Overexpression of p185 is also associated with resistance of certain human breast tumor cell lines to the cytotoxic effects of TNF-α.

In another embodiment of the invention, tumor cells are treated by (1) administering to a patient antibodies directed against the growth factor and/or its receptor, that inhibit the biological function of the receptor and that sensitize the cells to cytotoxic factors such as TNF, and (2) administering to the patient cytotoxic factor(s) or other biological response modifiers which activate immune system cells directly or indirectly to produce cytotoxic factors.

The cytotoxic factor, such as TNF-α, exerts its cytostatic (cell growth suppressive) and cytotoxic (cell destructive) effect. Examples of useful cytotoxic factors are TNF-α, TNF-β, IL-1, IFN-γ and IL-2, and chemotherapeutic drugs such as 5FU, vinblastine, actinomycin D, etoposide, cisplatin, methotrexate, and doxorubicin. Cytotoxic factors can be administered alone or in combination. In a still further embodiment of the invention, the patient is treated with antibodies which inhibit receptor function, and with autologous transfer therapy, e.g. LAK or TIL cells.

Tumor necrosis factors are polypeptides produced by mitogen-stimulated macrophages or lymphocytes which are cytotoxic for certain malignantly transformed cells. The anti-tumor effect of TNF-α is known to be synergistically potentiated by interferons. The anti-tumor effect of TNF-α and TNF-β in admixture are additive, as are the antiviral effects of interferons alpha and beta.

The tumor necrosis factors include TNF-α and TNF-β. The former is described together with methods for its synthesis in recombinant cell culture, in U.S. Patent 4,650,674, and in European Patent Application 0168214; the latter is described in European Patent Application 0164965. The TNF-α and TNF-β described in these patent documents includes cytotoxic amino acid sequence and glycosylation variants. TNF-α and TNF-β from non-recombinant sources are also useful in the method of this invention.

The preferred TNF is mature human TNF-α from recombinant microbial cell culture. The TNF ordinarily will have a cytolytic activity on susceptible L-M murine cells of greater than about 1 x 10^6 units/mg, wherein a unit is defined as set forth in the above-described patent application.

In another embodiment of the subject invention, one or more additional cytokines and/or cytotoxic factors are administered with TNF-α, egs. interferons, interleukins, and chemotherapeutic drugs.

The compositions herein include a pharmaceutically acceptable vehicle such as those heretofore used in the therapeutic administration of interferons or TNF, e.g. physiological saline or 5% dextrose, together with conventional stabilizers and/or excipients such as human serum albumin or mannitol. The compositions are provided lyophilized or in the form of sterile aqueous solutions.

Several variables will be taken into account by the ordinary artisan in determining the concentration of TNF in the therapeutic compositions and the dosages to be administered. Therapeutic variables also include the administration route, and the clinical condition of the patient.

The cytotoxic factor(s) and antibodies inhibiting growth factor receptor function are administered together or separately. If the latter, advantageously the antibodies are administered first and the TNF thereafter within 24 hours. It is within the scope of this invention to administer the TNF and antibodies in multiple cycles, depending upon the clinical response of the patient. The TNF and antibodies are administered by the same or separate routes, for example by intravenous, intranasal or intramuscular administration.

The method of the subject invention can be used with tumor cells which overexpress growth factor receptor and/or ligand where antibodies can be produced which inhibit the growth factor receptor function. A cell (e.g. breast tumor cell) overexpresses a growth factor receptor if the number of receptors on the cell exceeds the number on the normal healthy cell (e.g. normal breast tissue cell). Examples of carcinomas where the HER2 receptor is overexpressed (and thus the method of the subject invention is applicable), are human breast, renal, gastric and salivary gland carcinomas.

A further embodiment of the invention is an assay for identifying receptors and other proteins having increased tyrosine kinase activity, and for identifying oncogenes that transform cells. Amplification of certain oncogenes encoding tyrosine kinases correlates with TNF-α resistance. If cells are selected for resistance to TNF-α, some of these will have increased tyrosine kinase activity. Some of the tyrosine kinases will be receptors.